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Importance of soluble cytokine receptors for inflammation associated cancer

Ważna rola rozpuszczalnych receptorów cytokinowych w powstawaniu nowotworów związanych z zapaleniem

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Cytokine receptors, which exist in membrane-bound and soluble form, bind their ligands with comparable affinity. While most soluble receptors are antagonists in that they compete for the ligands with their membrane counterparts, some soluble receptors are agonists. In this case, the complex of ligand and soluble receptor binds on target cells to a second receptor subunit and initiates signal transduction. The soluble receptors of the IL-6 family of cytokines (sIL-6R, sIL-11R, sCNTF-R) are agonists. *In vivo*, the IL-6/soluble IL-6R complex stimulates several types of target cells not stimulated by IL-6 alone, since they do not express membrane-bound IL-6R. This process has been named transsignalling. We have shown that in several chronic inflammatory diseases such as chronic inflammatory bowl disease, peritonitis and rheumatoid arthritis, transsignalling via soluble IL-6R complexed to IL-6 is a crucial point in the transition from the acute to the chronic state of the disease. The mechanism by which the IL-6/ soluble IL-6R complex regulates the inflammatory state is discussed.

Key words: cytokine, cytokine receptor, soluble receptor, inflammation, gp130, sgp130Fc fusion protein.

Introduction

The Interleukin-6 (IL-6) family of cytokines acts via receptor complexes that contain at least one subunit of the signal transducing protein gp130 [1]. The family comprises IL-6, IL-11, ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), leukaemia inhibitory factor (LIF), and oncostatin M (OSM) [1]. IL-6, IL-11, and CNTF first bind to specific receptors, and these complexes associate with a homodimer of gp130 in the case of IL-6 and IL-11 or, alternatively, with a heterodimer of gp130 and the related protein LIF receptor (LIF-R) in the case of CNTF. OSM and LIF first bind directly to gp130 and LIF-R, respectively, and form heterodimers with LIF-R and gp130. Recently, a gp130-related protein was described that can heterodimerize with gp130 and that acts as an alternative OSM receptor [2]. CT-1 binds directly to the LIF-R and induces gp130/LIF-R heterodimer formation [3]. Recently, the presence of a specific glycosylphosphatidylinositol (GPI)-anchored CT-1 receptor on neuronal cells was implicated [3].

On target cells IL-6 first binds to the IL-6 receptor (IL-6R). The complex of IL-6 and IL-6R associates with the signal-transducing membrane protein gp130, thereby inducing its dimerization and initiation of signalling [1, 4]. gp130 is expressed by all cells in the body, whereas IL-6R is mainly expressed by hepatocytes, monocytes/macrophages and some lymphocytes. A naturally occurring soluble form of IL-6R (sIL-6R), which has been found in various body fluids, is generated by two independent mechanisms, limited proteolysis of the membrane protein and translation from an alternatively spliced mRNA [5-10]. Interestingly, sIL-6R together with IL-6 stimulates cells which only express gp130 [11-13], a process which has been named transsignalling [6, 7, 14]. Recently, it has been shown that sIL-6R strongly sensitizes target cells [15]. Embryonic stem cells [16, 17], early haematopoietic progenitor cells [14, 18], many neural cells [19, 20], smooth muscle cells [21] and endothelial cells [22], among others, are only responsive to IL-6 in the presence of sIL-6R [23].

Most cytokine receptors exist in membrane-bound and soluble form. Interestingly, cytokines bind to both receptor forms with comparable affinity. While most soluble receptors are antagonists in that they compete with their membrane counterparts for ligands, some soluble receptors are agonists. In this case, the complex of ligand and soluble receptor binds on target cells to a second receptor subunit and initiates signal transduction. Soluble receptors of the IL-6 family of cytokines are agonists [7, 24, 25]. *In vivo*, the IL-6/sIL-6R complex stimulates several types of target cells, which are not stimulated by IL-6 alone, since they do not express membrane-bound IL-6R. Such cells include embryonic stem cells [16, 17], endothelial cells [22], haematopoietic progenitor cells [26, 27], osteoclasts [28] and neuronal cells [29, 30]. The fact

Obie formy receptorów cytokinowych (zarówno związana z błoną komórkową, jak i rozpuszczalna) wykazują podobne powinowactwo do swoich ligandów. Większość receptorów rozpuszczalnych ma działanie antagonistyczne w stosunku do ich form związanych z błoną komórkową, jednakże niektóre z nich pełnią rolę agonistów. W tym przypadku kompleks ligand-receptor rozpuszczalny jest wiązany przez komórkę docelową, gdzie łączy się z inną podjednostką receptorową, co powoduje inicjację przekazywania sygnału do wnętrza komórki. Rozpuszczalne receptory cytokin z rodziny IL-6 (sIL-6R, sIL-11R, sCNTF-R) są agonistami. W warunkach *in vivo* kompleks IL-6/sIL-6R stymuluje komórki, które nie wykazują ekspresji receptora IL-6 na swojej powierzchni i przez to są wyjściowo niewrażliwe na działanie samej IL-6. Proces ten jest określany mianem transsygnalingu. Autorzy pracy wykazali, że w przypadku kilku przewlekłych chorób zapalnych, takich jak zapalenia jelit, zapalenie otrzewnej i reumatoidalne zapalenie stawów, transsygnaling za pośrednictwem kompleksu IL-6/sIL-6R jest kluczowy w procesie przekształcenia zapalenia ostrego w jego postać przewlekłą. W pracy przedyskutowano mechanizm, za pośrednictwem którego omawiany kompleks IL6/sIL-6R reguluje reakcję zapalną.

Słowa kluczowe: cytokina, receptor cytokinowy, receptor rozpuszczalny, zapalenie, gp130, białko fuzyjne sgp130Fc.

that IL-6/sIL-6R promotes wound healing strongly argues for the fact that also keratinocytes are subject to transsignalling processes [31].

Interestingly, we could recently show that CNTF not only acts via membrane-bound or soluble CNTF-R. CNTF can also use membrane-bound and soluble IL-6R [32]. This fact might have important implications for the use of CNTF as a therapeutic agent. The use of CNTF as a drug in amytrophic lateral sclerosis (ALS) had to be discontinued due to severe peripheral side effects. This was surprising since the receptor for CNTF is not expressed outside of the central nervous system. The fact that CNTF can also signal via IL-6R may explain these side effects and may be the basis for the construction of CNTF variants which only bind to CNTF-R but not to IL-6R [32].

The concept of designer cytokines

Using the structural information available on membrane-bound and soluble cytokine receptors, we have constructed chimeric proteins in which receptor recognition modules have been altered or exchanged and in which cytokines have been fused to their soluble cytokine receptors. Furthermore, chimeric receptor proteins have been constructed which contain cytokine binding modules of gp130, LIFR or OSMRß. This approach has allowed the definition of cytokine binding modules on receptor proteins [33-36].

Furthermore, we have constructed a fusion protein consisting of the domains of IL-6 and sIL-6R which are necessary for biological function. The two proteins are covalently connected by a flexible polypeptide linker (Fig. 1). The recombinant protein was folded correctly and showed biological activity. The fusion protein, which we call Hyper-IL-6, is 100-1000 times more active than the separate proteins IL-6 and sIL-6R. Many cells including haematopoietic

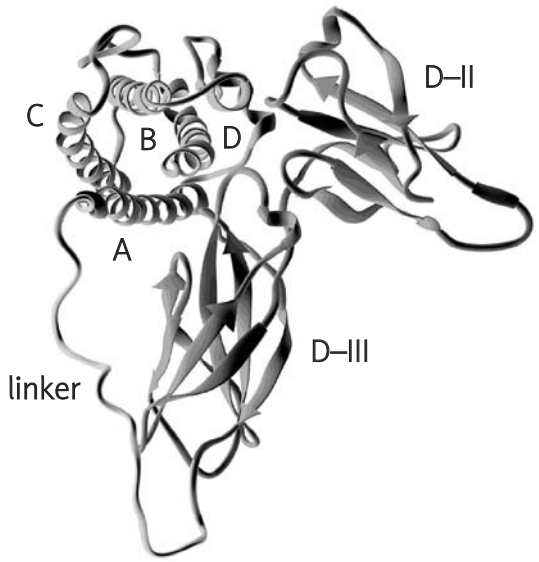


Fig. 1. Hyper-IL-6 is: a highly active designer cytokine consisting of IL-6 and soluble IL-6R. Molecular model of the fusion protein of IL-6 and sIL-6R (Hyper-IL-6)consisting of IL-6 and sIL-6R fused by a flexible peptide linker. A,B,C,D denote the four helices of IL-6; D-II and D-III are the two cytokine-binding receptor domains of the sIL-6R which were used for the construction of the fusion protein

Ryc. 1. *Hiper-IL6: wysoce aktywna zaprojektowana cytokina składająca się z IL-6 oraz rozpuszczalnej formy IL-6R. Molekularny model białka fuzyjnego IL-6 oraz sIL-6R (Hiper-IL-6) składającego się z IL-6 i sIL-6R połączonych elastycznym łańcuchem peptydowym. A,B,C,D – cztery helisy IL-6; D-II i D-III – dwie domeny receptora wiążącego cytokinę, które zostały użyte do konstrukcji białka fuzyjnego*

progenitor cells, neuronal cells, endothelial cells and smooth muscle cells which do not respond to IL-6 alone show a remarkable response to IL-6/sIL-6 R [23, 27, 30, 37, 38]. Recently, our approach has been adopted to construct a fusion protein between IL-11 and the soluble IL-11R [39]. A designer cytokine consisting of CNTF fused to the soluble CNTF-R was shown to exhibit high neurotrophic activity on primary hippocampal neurons [30].

Viral interleukin-6

The genome of HHV8 codes for several proteins with significant homologies to human antiapoptotic proteins, chemokines, and cytokines including a viral form of Interleukin-6 (vIL-6) with 25% homology to human IL-6 [40, 41]. vIL-6 has been demonstrated to have biologic activities reminiscent of human IL-6, i.e. stimulation of proliferation of murine hybridoma and human myeloma cells [40, 42, 43]. More recently it was shown in mice, injected with vIL-6 transfected NIH3T3 cells, that vIL-6 induced angiogenesis and haematopoiesis. It was concluded that through these functions vIL-6 played an important role in the pathogenesis of HHV8-associated disorders [44].

We have recently shown that purified recombinant vIL-6 directly binds to gp130 and stimulates primary human smooth muscle cells and primary human Kaposi sarcoma cells. IL-6R fails to bind vIL-6 and is not involved in its signalling. Our data demonstrate that vIL-6 is the first cytokine which directly binds and activates gp130. This property points to a possible role of this viral cytokine in the pathophysiology of HHV8 [45-48]. In Fig. 2 we show the vIL-6 stimulation of HepG2 cells which have been engineered to express no IL-6R on the cell membrane [12]. On these cells, human IL-6 does not lead to STAT3 activation, whereas vIL-6 and Hyper-IL-6 activate STAT3 activity. The activation of STAT3 can be completely inhibited

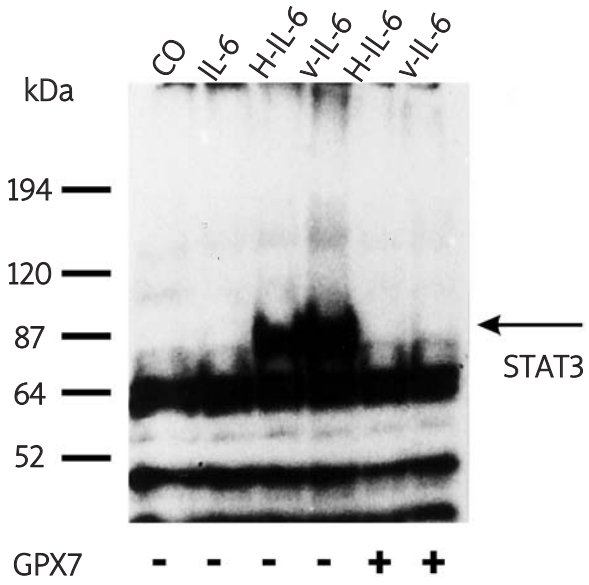


Fig. 2. STAT3 activation by vIL-6 via direct stimulation of gp130 on human hepatoma cells. HepG2-IL-6 cells were stimulated with 100 ng/ml IL-6, HyperIL-6, and vIL-6 in the presence or absence of the neutralizing gp130 mAB GPX7 (1 mg/ml) for 15 min. Cells were lysed and proteins were separated by SDS-PAGE and blotted onto nitrocellulose. Phosphorylated STAT3 protein was detected by Western blotting using a phosphospecific STAT3 mAB

Ryc. 2. *Aktywacja STAT3 przez vIL-6 za pomocą bezpośredniej stymulacji gp130 w komórkach ludzkiego hepatoma. Komórki HepG2-IL-6 były poddawane przez 15 min działaniu 100 ng/ml IL-6, Hiper-IL-6 i vIL-6 w obecności lub przy braku przeciwciała monoklonalnego GPX7 (1 mg/ml), które neutralizuje gp130. Komórki poddawano lizie i białka były rozdzielane przez SDS-PAGE, a następnie transferowane na nitrocelulozę. Fosforylowana forma STAT3 była wykrywana metodą Western-blottingu z użyciem fosfo--STAT3 mAB*

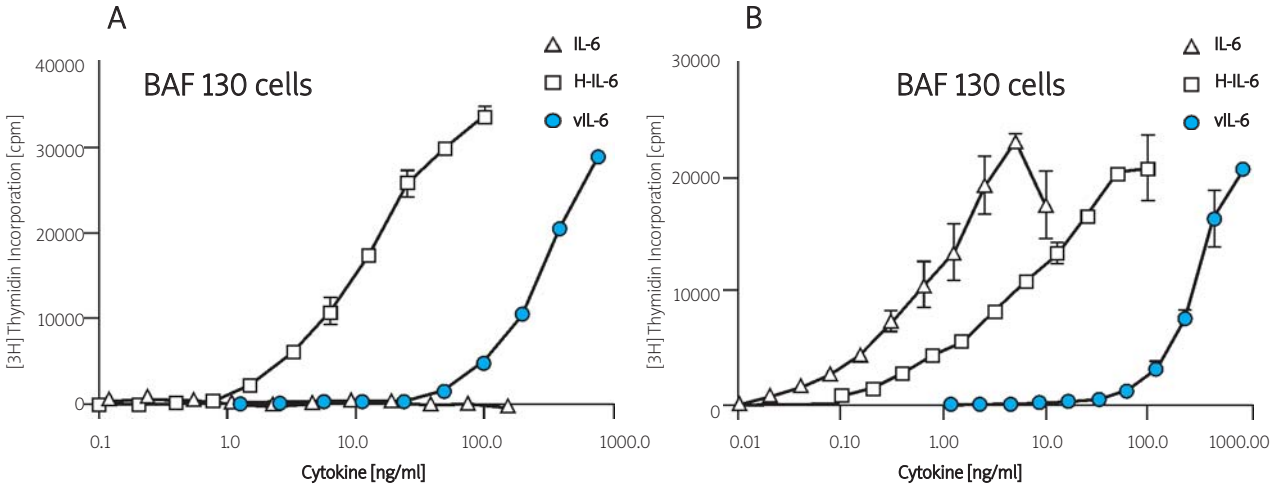


Fig. 3. Biological activity of vIL-6 is mediated by gp130 directly. BAF/3 cells stably transfected with a human gp130 cDNA (A) and BAF/3 cells stably transfected with human gp130 and IL-6R cDNAs (B) were stimulated with increasing amounts of HyperIL-6 (H-IL-6), human IL-6, and vIL-6. Proliferation of the cells was assessed by measuring [3H]-thymidine incorporation into DNA

Ryc. 3. *Aktywność biologiczna vIL-6 jest mediowana bezpośrednio przez gp130. Komórki BAF/3 stabilnie transfekowane cDNA ludzkiego gp130 (A) oraz komórki BAF/3 stabilnie transfekowane cDNA ludzkiego gp130 i IL-6R (B) były poddawane działaniu wzrastających ilości Hiper-IL-6 (H IL-6), ludzkiej IL-6 oraz vIL-6. Proliferacja komórek była oceniana poprzez pomiar wbudowywania [3H]-tymidyny do DNA*

by a neutralizing gp130 antibody (Fig. 2). As can be seen in Fig. 3, vIL-6 stimulates the proliferation of BAF/3 cells, which only express gp130 but not IL-6R (Fig. 3A). The presence of IL-6R in BAF/3 cells does not lead to a change in the observed dose response curve, indicating that IL-6R is not used by the viral cytokine (Fig. 3B). We have recently generated scFv antibody against the vIL-6 protein, which was shown to have neutralizing properties. This antibody was also exploited in the form of an intracellular intrabody, which was anchored in the ER of vIL-6 synthesizing cells. By this strategy, secretion of vIL-6 by such cells could be completely abrogated [60]. We believe this might be an example for a new strategy to neutralize virus encoded proteins involved in the pathophysiology of these agents [60].

The fact that vIL-6 forms a functional complex with gp130 without the need for IL-6R [45, 46] has been exploited to crystallize the complex of the extracellular portion of gp130 together with vIL-6. This led to the first structural information of a member of the complex type cytokines together with its receptor [49]. Complex type cytokines require interaction with three cytokine receptor subunits to induce cellular signalling. In the case of IL-6 there is an interaction of the cytokine with IL-6R and two molecules of gp130. In the case of CNTF the cytokine would interact with CNTF-R, gp130 and the LIF-R protein [1]. Members of this group of cytokines comprise besides IL-2 and IL-15 several members of the gp130 cytokine family such as IL-6, IL-11, CNTF, CT-1 and CLC. Structural information on the simple type cytokine family which comprises (among others) growth hormone and prolactin has been available for more than 10 years [50].

The role of soluble GP130

The role of a soluble form of gp130 (sgp130) was analyzed using two soluble gp130 fusion proteins. In the first version, the extracellular portion of gp130 was fused to a COOH-terminal hexa-histidine tag. In a second version, the extracellular portion of gp130 was fused to the constant portion of a human IgG1 antibody protein. As can be seen in Fig. 4, sgp130 only inhibited the expression of the acute phase protein antichymotrypsin (ACT) in HepG2 cells, which had been treated with Hyper-IL-6. The induction of acute phase protein expression in HepG2 cells by human IL-6 is unaffected by soluble gp130 (Fig. 4B). It turned out that sgp130 exclusively inhibited IL-6 responses mediated by sIL-6R without interfering with responses via the membrane-bound IL-6R [51-54]. Therefore we postulated that sgp130 acts as a natural inhibitor of IL-6/sIL-6R complexes. Our model of the molecular mechanism by which soluble gp130 exerts specific inhibition towards the IL-6/sIL-6R complex is depicted in Fig. 5. IL-6 does not bind to soluble gp130. So IL-6 binds to the membrane-bound IL-6R and forms a complex with membrane-bound gp130. The soluble gp130 protein does not have access to this complex, which therefore is not inhibited (Fig. 5A). The IL-6/sIL-6R complex binds as well to the soluble and the membrane-bound gp130. Therefore, a molar excess of sgp130 leads to inhibition of the biologic response (Fig. 5B) [55].

A functional role of sIL-6R has recently been demonstrated in chronic inflammatory bowl disease (Crohn's disease). We could show that T-cells of Crohn's disease patients are extremely resistant to apoptosis and show activation of the JAK-STAT signal transduction pathway. These T-cells produce large amounts of IL-6 but lack membrane-bound IL-6R. Surprisingly, treatment of these cells with a neutralizing monoclonal antibody to IL-6R induced apoptosis. Moreover, treatment of the cells with sgp130 showed the same effect (Fig. 6). These results clearly demonstrate that IL-6 is involved in apoptotic resistance of T-cells of Crohn's disease patients. Moreover, the data demonstrate that sIL-6R and not the membrane-bound IL-6R is responsible for T-cell stimulation.

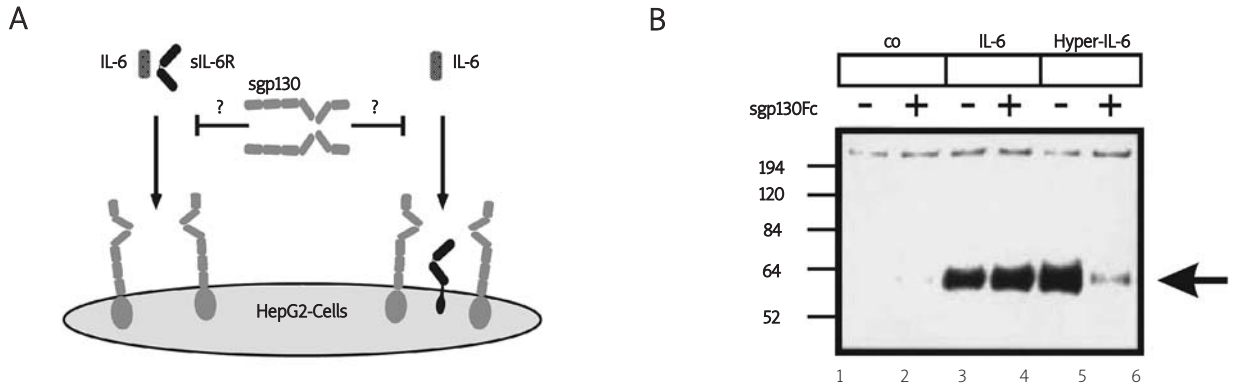


Fig. 4. Gp130 selectively inhibits signaling via IL-6/sIL-6R without affecting signaling via the membrane bound IL-6R. HepG2 cells were stimulated with IL-6 or IL-6/sIL-6R in the presence or absence of soluble gp130. (A) Schematic representation of the experiment. (B) The induction of the acute phase protein antichymotrypsin on HepG2 cells was only inhibited by soluble gp130 when the cells were stimulated with IL-6/sIL-6R but not with IL-6 alone. These data indicate that sgp130 selectively inhibits biologic responses of IL-6/sIL-6R but not of IL-6 acting on the membrane bound IL-6R

Ryc. 4. *Gp130 wybiórczo hamuje przekazywanie sygnału przez IL6/sIL-6R bez wpływu na przekazywanie sygnału przez związany z błoną IL-6R. Komórki HepG2 były poddawane działaniu IL-6 lub IL-6/sIL-6R w obecności lub nieobecności rozpuszczalnego gp130. (A) Schemat doświadczenia. (B) Indukcja białka ostrej fazy antychymotrypsyny w komórkach HepG2 była hamowana przez rozpuszczalne gp130, gdy komórki były poddawane działaniu IL-6/sIL-6R, ale nie wtedy, gdy poddawano je działaniu samej IL-6. Te dane wskazują, że sgp130 wybiórczo hamuje odpowiedź biologiczną na działanie IL-6/sIL-6, ale nie na działanie IL-6 wiążącej się z błonowym IL-6R*

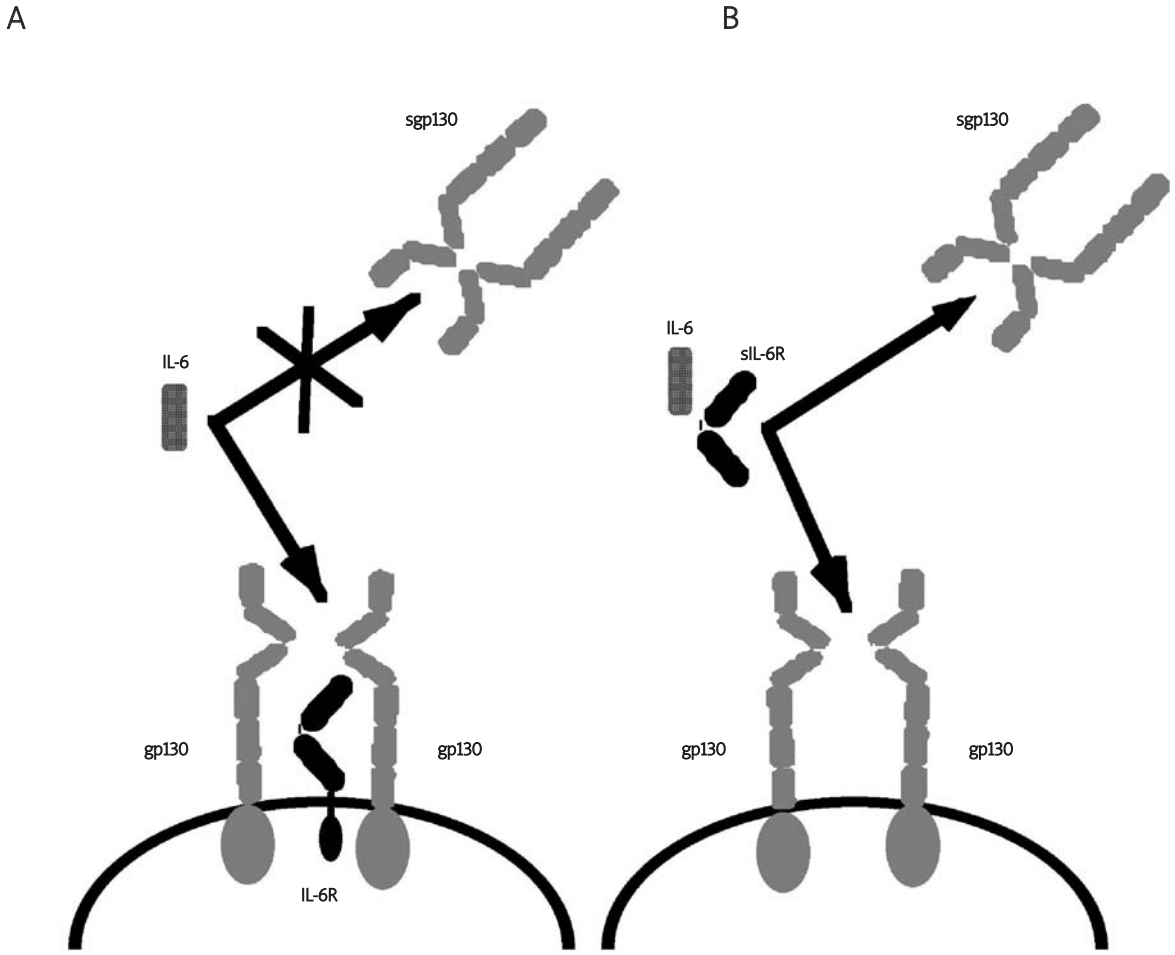
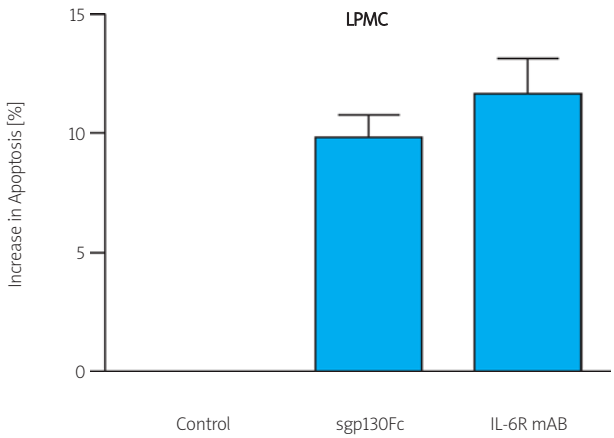


Fig. 5. Schematic view of the inhibitory mechanism of sgp130. (A) sgp130 has no access to IL-6 complexed by membrane bound IL-6R and two molecules of membrane bound gp130. (B) The IL-6/sIL-6R complex can bind to both, membrane bound and sgp130. Consequently, a molar excess of sgp130 leads to competitive inhibition of the IL-6/sIL-6R response

Ryc. 5. *Schemat mechanizmu hamującego działania sgp130. (A) sgp130 nie ma dostępu do IL-6 związanej przez błonowy IL-6R i dwie cząsteczki błonowego gp130. (B) Kompleks IL-6/sIL-6R może wiązać się z obiema formami – związanym z błoną i rozpuszczalnym gp130. W konsekwencji nadmiar molarny sgp130 prowadzi do kompetytywnego hamowania odpowiedzi na działanie kompleksu IL-6/sIL6R*

Fig. 6. Apoptosis of lamina propria mononuclear cells (LPMC) of Crohn's disease patients upon treatment with sgp130. LPMCs were isolated and cultured for 48 h in the presence or absence of 10 µg/ml of a neutralizing mAB specific for human IL-6R or 10 µg/ml sgp130Fc. Subsequently, cells were stained for annexin V and propidiumiodide and analyzed by FACS. The increase in apoptotic (annexin V positive and propidiumiodide negative) cells is shown. The data presented are means of triplicate measurements with standard errors shown as vertical bars

Ryc. 6. *Apoptoza komórek jednojądrzastych blaszki właściwej (LPMC) pacjentów z chorobą Crohna poddanych działaniu sgp130. LPMC były izolowane i hodowane przez 48 godz. w obecności lub nieobecności 10 µg/ml mAB specyficznego dla ludzkiego IL-6R lub 10 µg/ml sgp130Fc. Następnie komórki były barwione na obecność aneksyny V i propidiumiodide oraz analizowane metodą FACS. Pokazano wzrost liczby komórek apoptotycznych (aneksyna V-pozytywnych i propidiumiodide-negatywnych). Prezentowane dane są średnią z trzykrotnego powtórzenia pomiarów, wraz z błędami standardowym pokazanymi jako pionowe słupki*



Most likely, sIL-6R is produced by lamina propria macrophages or neutrophils [51, 52].

The fact that in Crohn's disease the chronic inflammatory state is maintained with the help of IL-6/sIL-6R signalling seems to be a more general phenomenon. It was recently shown in a murine Peritonitis model that the transition between the acute phase, which is governed by neutrophils, to the chronic state, which is characterized by massive mononuclear cell infiltration, is regulated by the level of the soluble IL-6R complex present in the peritoneum. The sIL-6R in the peritoneum is presumably generated by shedding from neutrophilic cells. Therefore the transition of the neutrophil to the mononuclear cell phase could be inhibited by the addition of soluble gp130 protein [53].

An additional impressive example of the therapeutic potential of the sgp130 protein was the recent demonstration that the course of a murine model of monoarthritic Ag-induced arthritis [54, 58] and murine colitis and colon cancer [56, 57, 59] could be blocked by this protein. The feasibility of a therapeutic application of sgp130 is currently being considered.

Conclusion

We conclude that sgp130 is the natural inhibitor of IL-6 responses which are dependent on sIL-6R. Furthermore, recombinant sgp130 is expected to be a valuable therapeutic tool to specifically block disease states in which sIL-6R transsignalling responses exist, e.g. in Crohn's disease and other chronic inflammatory diseases.

Note

There are inclusions in this text from a number of different articles which are cited in the reference section.

Acknowledgments

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